Adaptive Regulation of Ascorbic Acid Synthesis in Rat-Liver Extracts

EFFECT OF X-IRRADIATION AND OF DIETARY CHANGES

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It has been observed by Caputto, McCay & Carpenter (1958) that the synthesis of ascorbic acid from glucuronic acid was impaired in rat-liver extracts from vitamin E-deficient rats. This was attributed to an inhibitory effect of lipid peroxides formed *in vitro* by liver preparations from vitamin E-deficient animals (Carpenter, Kitabchi, McCay & Caputto, 1959). Since it is known that excess of lipid peroxides are formed *in vivo* (Bacq, Burg, Chavallier & Heugsheim, 1951; Horgan & Philpot, 1954; Mal'ts, 1960) and *in vitro* (Bernheim, Ottolenghi & Wilbur, 1956; Barber & Wilbur, 1959) after X-irradiaton, this investigation was undertaken to detect any impairment of ascorbic acid synthesis in liver extracts from X-irradiated rats, as a consequence of increased formation of fatty peroxides. It was observed that preparations from irradiated animals formed less ascorbic acid than those from normal rats; however, this appeared to be a consequence of the decreased food intake after irradiation. This observation led us to study the effect of starvation and of dietary changes on the activity of the enzymic system synthesizing ascorbic acid.

EXPERIMENTAL

Materials

Chemicals. ATP and NAD were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), D-glucuronolactone and L-ascorbic acid from Merck AG (Darmstadt, Germany), and 2-thiobarbituric acid from Sigma Chemical Co. or from Fluka AG (Buchs, Switzerland). Other chemicals were of analytical grade. Solutions were prepared in de-ionized water.

Animals. Male rats of the Wistar–Glaxo strain inbred in our Institute were used, when about 2 months old and weighing about 140 g. All animals were housed in an air-conditioned room at 20–24°.

Methods

Dietary treatment. Rats were fed from weaning with a commercial diet (Diet Zeta, Zoofarm, Padova), containing 17.2% of protein and 40 mg. of vitamin E/kg. (manufacturer's data). The semi-synthetic control diet was the 20% casein diet A described by Bonetti & Stirpe (1962). The carbohydrate-free diet S1 was derived from diet A by substituting casein for starch and sucrose and preparing the vitamin premix with casein. Vitamin E (Ephynal, Prodotti Roche) was added at the excess concentration of 500 mg./kg. (Corwin & Schwarz, 1959) in all semi-synthetic rations. Food was given to animals as dry powder in conical containers to prevent spillage. Forcibly fed rats received daily 12–15 g. of diet A with enough water added to obtain a slurry. This was given in three doses, through a plastic stomach tube attached to a syringe. Rats on semi-synthetic rations received diet A for 4–5 days before being killed or irradiated; to compensate for any possible effect of sudden radical changes in the type of ration, rats on diet S1 were pre-accustomed to the semi-synthetic diet, being fed with diet A for 4–5 days. Unless otherwise stated, all rats received food and water *ad libitum*.

Irradiation. Rats were exposed to whole-body irradiation from a 200 kv X-ray apparatus (Stabilipan, Siemens) through a 1 mm. Cu half-value layer, and at a focus-subject distance of 40 cm. Total dose was 600 or 800 r., given at the rate of 80 r./min. To ensure correct positioning during exposure, the rats were constrained, two at a time, in a thin wooden box with two narrow body-size compartments.

Ascorbic acid synthesis. This was determined as described by ul Hassan & Lehninger (1956). The rats were killed and the livers were rapidly removed, washed in ice-cold 0.15M-KCl, weighed and homogenized in 0.15M-KCl (2.5 ml./g. of liver) in a Potter-type homogenizer fitted with a Teflon pestle (A. H. Thomas Co., Philadelphia, Pa., U.S.A.), with six to seven complete up-and-down movements. The homogenates were centrifuged for 10 min. at 3000g at 0° in a Martin–Christ refrigerated centrifuge fitted with multispeed attachment and rotor 5070. The assay system contained: 50μmoles of potassium phosphate buffer, pH 7.4, 4μmoles of NAD, 4μmoles of ATP, 75μmoles of nicotinamide, 10μmoles of MgCl$_2$, 10μmoles of D-glucuronolactone and 0.7 ml. of the supernatant (hereafter called liver extract), in a final volume of 2.5 ml. The mixtures were incubated in conical flasks in a Dubnoff shaker at 37° in air. The reaction was stopped after 2 hr. with 2.5 ml. of 16% (w/v) trichloroacetic acid. Zero-time controls were run by adding trichloroacetic acid before the liver extract. The mixtures were centrifuged and samples (1–2 ml.) of the supernatant used for the determination of 'total' ascorbic acid by the method of Roe & Kuether (1943), with bromine water as oxidizing agent and the incubation procedure of Geschwind, Williams & Li (1951).

The colour formed was read in a Uvispek spectrophoto-meter at 520 mμ; readings of the zero-time controls were subtracted from those of the corresponding incubated samples. In preliminary experiments it was checked that formation of ascorbic acid was linearly related to time and to the amount of liver extract used.

Ascorbic acid in liver and urine. For the determination of the ascorbic acid content of the liver, 2·0 ml. of the liver homogenate was added, immediately after preparation, to an equal volume of 16% trichloroacetic acid and centri-fuged. Ascorbic acid was estimated on samples of the supernatant by the same procedure as was used for the enzymic assays.

Rats were isolated 24 hr. before being killed in metabolic cages over a funnel with a glass-wool plug. Urine was collected under liquid paraffin and brought to a known volume with the washings of the funnel. The ascorbic acid content was determined by the method of Roe & Kuether (1943) as described by Roe (1954).

Formation of lipid peroxides. Samples (0·5 ml.) of the liver extract were shaken at 37° with 1·5 ml. of 0·15 M-potassium phosphate buffer, pH 7·4. After 1 hr. 2·0 ml. of 10% trichloroacetic acid was added and the mixtures were centrifuged. Peroxides formed were determined in 2·0 ml. of the supernatant by the method of Tappel & Zalkin (1959) as used by Bieri & Anderson (1960). In some experi-ments the colour was stabilized by bringing the mixture to pH 12·0–12·5 with KOH, as done by Pricer & Horecker (1960).

Nitrogen determination. This was performed on samples of the liver extract by the micro-Kjeldahl method, by digesting with the catalyst of Chibnall, Rees & Williams (1943) and distilling in a Markham (1942) apparatus.

RESULTS

Effect of irradiation and of reduced food intake. Experiments were begun with rats on the stock diet. After irradiation of rats with 800 r., the synthesis of ascorbic acid was considerably re-duced (Table 1); a similar reduction was observed in normal rats paired-fed with the irradiated ones.

Since it had been observed by E. Bonetti, G. Caprino & F. Stirpe (unpublished work) that a semi-synthetic diet was better accepted by X-irradiated rats, experiments were repeated with rats on diet A. Formation of peroxides was in-hibited by addition of a large excess of vitamin E to this diet. The same effect of irradiation was observed in these animals (Table 1); the synthesis of ascorbic acid was less depressed in irradiated rats than in their paired-fed controls. An attempt was made to overcome the effect of anorexia by feeding some rats forcibly after irradiation, but without any improvement in the rate of synthesis being obtained. One of these rats died 3 days after irradiation, whereas no mortality was observed in irradiated rats fed *ad libitum*. This animal, as well the survivors of the same group, showed the patho-logical picture of the gastrointestinal tract de-scribed by Smith, Ackermann & Smith (1952), and therefore it must be concluded that the food given was not utilized.

Table 1. *Effect of X-irradiation and of decreased food intake on synthesis, liver content and urinary excretion of ascorbic acid, and on formation of lipid peroxides by rat-liver extracts*

Peroxides were estimated as described by Bieri & Anderson (1960). Numbers of animals used are given in parentheses and results are expressed ± S.E.M.

| | Ascorbic acid | | | |
	Formed *in vitro* (μmole/mg. of N/2 hr.)	Liver content (μmoles/g. wet wt.)	Excreted in urine (μmoles/24 hr.)	Peroxides formed/hr. ($E_{535\,m\mu}$)
Treatment				
Rats on stock diet				
Control (4)	0·164 ± 0·009	1·45 ± 0·14	1·31 ± 0·08	0·223 ± 0·008
2 days after 800 r. (4)	0·058 ± 0·019	1·50*	2·02*	0·314 ± 0·050
Paired-fed† (2)	0·042	1·79	1·22	0·348
4 days after 800 r. (4)	0·002‡	0·87*	1·64*	0·341*
Paired-fed† (2)	0·029	1·46	1·14	0·384
Rats on semi-synthetic diet A				
Control (8)	0·174 ± 0·018	1·77 ± 0·09	1·98 ± 0·25 ⎫	
2 days after 600 r. (4)	0·106 ± 0·033	1·92 ± 0·08	1·64 ± 0·17 ⎪	
Paired-fed† (4)	0·046 ± 0·004	1·72 ± 0·07	0·83 ± 0·21 ⎪	
4 days after 600 r. (4)	0·137 ± 0·023	1·50 ± 0·11	1·48 ± 0·18 ⎪	
Paired-fed† (4)	0·101 ± 0·022	1·64 ± 0·23	1·44 ± 0·24 ⎪	
6 days after 600 r. (4)	0·100 ± 0·021	1·37 ± 0·17	1·91 ± 0·28 ⎬ Absent	
Paired-fed† (4)	0·073 ± 0·013	1·44 ± 0·24	1·99 ± 0·13 ⎪	
4 days after 800 r. (4)	0·037 ± 0·003	1·66 ± 0·07	0·28 ± 0·06 ⎪	
Paired-fed† (3)	0·031 ± 0·007	1·43 ± 0·07	0·45 ± 0·12 ⎪	
4 days after 800 r. (3) (forcibly fed)	0·041 ± 0·003	1·75 ± 0·11	— ⎭	

* Two animals only (other determinations not made).
† All paired-feeding experiments refer to the preceding group.
‡ This results from a value of 0·010 obtained in one rat, the other ones being 0·000.

Effect of starvation and subsequent feeding. The effect of starvation was then investigated, and it appeared that a 24 hr. period of starvation was enough to decrease the synthesis of ascorbic acid (Table 2). Since Caputto et al. (1958) had shown that the maximum effect of vitamin-E deficiency on the synthesis of ascorbic acid was reached as shortly as 3–4 days after deprivation, the possibility was considered that the effect of starvation was actually due to lack of vitamin E. This was discounted by giving starved animals enough vitamin E to prevent formation of peroxides; there was no effect on the synthesis of ascorbic acid. The effect of starving was quickly reversed by feeding the rats again for 24 hr.

The content of ascorbic acid in the liver decreased in starved animals, although not to such an extent as the synthesis, and not in starved rats supplemented with vitamin E. The amounts in urine were very irregular in these animals. The

Table 2. *Effect of starvation and resumed feeding on synthesis, liver content and urinary excretion of ascorbic acid, and on formation of lipid peroxides in rats on stock diet*

Peroxides were estimated as described by Bieri & Anderson (1960); the colour was stabilized by the method of Pricer & Horecker (1960). Numbers of animals used are given in parentheses and results are expressed ± s.e.m.

| | Ascorbic acid | | | |
| | Formed *in vitro* (μmole/mg. of N/2 hr.) | Liver content (μmoles/g. wet wt.) | Excreted in urine (μmoles/24 hr.) | Peroxides formed/hr. ($E_{543\,m\mu}$) |
Treatment				
Control (6)	0·151±0·008	1·81±0·13	2·51±0·82	0·132±0·075
Starvation for 24 hr. (4)	0·035±0·002	1·58±0·11	2·92±1·15	0·402±0·061
Starvation for 48 hr. (4)	0·043±0·009	1·52±0·06	—	0·095±0·039
Starvation for 24 hr. + vitamin E* (4)	0·030±0·004	2·12±0·13	1·72±0·23	Absent
Feeding for 24 hr. after 24 hr. starvation (4)	0·149±0·002	1·76±0·06	1·91±0·35	0·151±0·039

* 9 mg. of vitamin E was given subcutaneously in three doses, 24, 14 and 2·5 hr. before death.

Table 3. *Effect of carbohydrate-free diet and of administration of glucose, glucuronolactone and ascorbic acid on the synthesis, liver content and urinary excretion of ascorbic acid*

Peroxides were estimated as described in Table 2. Numbers of animals used are given in parentheses and results are expressed ± s.e.m.

| | Ascorbic acid | | | |
| | Formed *in vitro* (μmole/mg. of N/2 hr.) | Liver content (μmoles/g. wet wt.) | Excreted in urine (μmoles/24 hr.) | Peroxides formed/hr. ($E_{543\,m\mu}$) |
Treatment				
Rats on semi-synthetic diets				
Control* (8)	0·174±0·018	1·77±0·09	1·98±0·25	Absent
Carbohydrate-free for 24 hr. (8)	0·077±0·005	1·38±0·19	2·39±0·47	
Carbohydrate-free for 6 days (8)	0·112±0·009†	1·37±0·18	3·95±0·57‡	
Rats on stock diet				
Control§ (6)	0·151±0·008	1·81±0·13	2·51±0·82	0·132±0·075
Control + 300 mg. of glucuronolactone‖ (4)	0·160±0·015	2·41±0·14	3·09±0·14	0·068±0·026
Starvation for 24 hr. + 300 mg. of glucuronolactone‖ (4)	0·047±0·003	2·12±0·08	3·58±0·72	0·146±0·014
Starvation for 24 hr. + 400 mg. of glucuronolactone¶ (4)	0·058±0·003**	2·25±0·18	5·29±0·64	0·197±0·013
Starvation for 24 hr. + 400 mg. of glucose¶ (4)	0·053±0·004**	1·42±0·05	1·50±0·15	0·256±0·066
Control + 400 mg. of ascorbic acid¶ (4)	0·235±0·044††	4·10±0·03	—	0·154±0·010

* See Table 1.
† Significantly different ($P < 0\cdot01$) from control and from carbohydrate-free for 24 hr.
‡ Seven animals only (other determinations not made).
§ See Table 2.
‖ 100 mg. intraperitoneally at 24, 16 and 2 hr. before death.
¶ 100 mg. intraperitoneally at 24, 16 and 2 hr. before death, and 100 mg. subcutaneously at 2 hr.
** Significantly different ($P < 0\cdot05$) from 24 hr. starvation (Table 2).
†† Significantly different ($P < 0\cdot05$) from control.

formation of peroxides was somewhat erratic, and was not correlated with the synthesis of ascorbic acid.

Effect of omission of carbohydrates from the diet and of administration of precursors. The effect of starvation could be attributed either to the stress or to the lack of some dietary components. A strong impairment of the synthesis of ascorbic acid was observed in rats given a carbohydrate-free diet for 24 hr., whereas values significantly higher but still below normal ones were obtained by giving this same diet for 6 days (Table 3). Rats on this ration had a lower content of ascorbic acid in the liver, but showed an enhanced excretion of ascorbic acid in the urine. Since carbohydrates are precursors of ascorbic acid in the rat, this observation led to the hypothesis of an adaptive response of the enzyme system to lack of substrates, and evidence was sought by giving glucuronolactone to rats. Administration of glucuronolactone did not affect the rate of synthesis in normal rats, but caused a moderate but significant enhancement in starved animals. However, a similar enhancement followed the administration of an equal amount of glucose. All rats receiving glucuronolactone had a higher liver content and an enhanced urinary excretion of ascorbic acid.

Ascorbic acid was given to normal rats to see if there was 'repression' of the enzyme by product. A stimulation of the synthesis was observed.

DISCUSSION

Liver extracts from X-irradiated rats did not form peroxides above the amount observed in their paired-fed controls, under conditions that are virtually the same as those of Carpenter *et al.* (1959). The impaired synthesis of ascorbic acid was caused mainly by the reduction of food intake consequent on irradiation. With one exception, the synthesis was actually more depressed in paired-fed controls than in irradiated animals, in spite of a more severe weight loss observed in the latter. Fitch, Chaikoff & Hill (1961) made a similar observation on a series of enzymes of carbohydrate metabolism in irradiated and non-irradiated rats starved for 24 hr. They suggested that irradiated animals have a reduced capacity of adaptation to the new metabolic condition imposed by starvation.

The effect of reduced food intake as well as of starvation (hereafter considered together) can be explained in several ways. The increase in formation of peroxides is ruled out, since the synthesis of ascorbic acid was decreased to the same extent even when formation of peroxides was abolished by administration of vitamin E.

The drastic fall in synthesis after a short period of deprivation of dietary carbohydrate is suggestive of an adaptation of the enzyme system under study caused by the lack of carbohydrates. A number of enzymes involved in carbohydrate metabolism have been shown to undergo adaptation after changes in the quality or in the quantity of carbohydrates in the diet (Freedland & Harper, 1957; Weber & Cantero, 1957; Weber, Banerjee & Bronstein, 1961; Fitch & Chaikoff, 1960; Fitch, Hill & Chaikoff, 1959; Di Pietro & Weinhouse, 1960; Niemeyer, González & Rozzi, 1961; Niemeyer, Pérez, Radojkovic & Ureta, 1962). The higher rate of synthesis observed after a longer period of carbohydrate deprivation would be compatible with this hypothesis. It is likely that after a period gluconeogenesis operates more efficiently than immediately after removal of carbohydrate from the diet, owing to the readjustment of the activity of other enzymes. Indirect support to this view is given by the observation that giving diet S1 for 24 hr. almost always caused a decrease of body weight of the rats; this was a transitory phenomenon, and could not be attributed to anorexia after the change of diet, since the food intake was the same. Therefore the enzyme system synthesizing ascorbic acid seems to respond to the lack of dietary carbohydrate with a 'primary adaptation' followed by a 'secondary adaptation', similar to the response of glucose 6-phosphatase to removal of glucose from the diet observed by Freedland & Harper (1958).

Administration of glucuronolactone had no more effect than that of glucose on the synthesis of ascorbic acid in starved rats. Hence it seems that the activity of synthesizing enzymes is regulated by dietary carbohydrates rather than by the amount of the more direct substrate.

Stimulated synthesis was observed after administration of ascorbic acid, and no explanation is offered for this, except that this compound may act as a drug rather than as the product of the reaction under study.

In most of our experiments the decreased synthesis was accompanied by a lower liver content and by a decreased urinary excretion of ascorbic acid. This concomitance would suggest that the impairment observed *in vitro* is the expression of a similar impairment occurring *in vivo*; additional support would be given by the lowered incorporation of ^{14}C from glucose into ascorbic acid observed by Loewus, Kelly & Hiatt (1960) in starved rats. However, the large amount of ascorbic acid present in the liver and urine of starved rats given glucuronolactone indicates that these animals are able to synthesize normal quantities of ascorbic acid, in spite of the low activity of the synthesizing enzymes, as measured by the assays *in vitro*. This, and the fact that the synthesis always decreases to a greater extent than the liver content and the

urinary excretion of the product, seems to mean that the enzymes responsible for the synthesis of ascorbic acid are present in the normal rat liver in large excess, and that even when reduced are still able to operate the synthesis at normal and even above normal rate. Boyland & Grover (1961) showed that some drugs enhanced the synthesis of ascorbic acid, as measured by the urinary excretion, by far more than the enzymic synthesis *in vitro* (from galactonolactone). From the rate of synthesis *in vitro*, these authors estimated that the activity of the enzymes is not the rate-limiting factor in the biosynthesis of ascorbic acid *in vivo*.

SUMMARY

1. The synthesis of ascorbic acid by rat-liver extracts is impaired after whole-body X-irradiation of the animals. This impairment is caused by the reduction of food intake after irradiation.

2. The synthesis of ascorbic acid is greatly reduced after starvation for 24 hr. Administration of glucuronolactone has little effect on the synthesis.

3. Feeding rats with a carbohydrate-free diet for 24 hr. causes a considerable reduction of ascorbic acid synthesis. A less severe reduction is observed if this diet is given for 6 days.

4. It is concluded that the enzymes synthesizing ascorbic acid are adaptive to dietary carbohydrates rather than to the direct substrate.

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